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—Original Article—

Canine decidualization *in vitro*: extracellular matrix modification, progesterone mediated effects and selective blocking of prostaglandin E2 receptors

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Abstract. Recently, we established an *in vitro* model with immortalized dog uterine stromal (DUS) cells for investigations into canine-specific decidualization. Their capability to decidualize was assessed with cAMP and prostaglandin (PG) E2. Here, we show that the effects of PGE2 are mediated through both of the cAMP-mediating PGE2 receptors (PTGER2/4). Their functional inhibition suppressed gene expression of *PRLR* and *PGR* in DUS cells. We also assessed the effects of cAMP and PGE2 on selected extracellular matrix components and CX43, and showed that cAMP, but not PGE2, increases COL4, extracellular matrix protein 1 (ECM1) and CX43 protein levels during *in vitro* decidualization, indicating a mesenchymal-epithelial decidual transformation in these cells. Thus, although PGE2 is involved in decidualization, it does not appear to regulate extracellular matrix. Further, the role of progesterone (P4) during *in vitro* decidualization was addressed. P4 upregulated *PRLR* and *PGR* in DUS cells, but these effects were not influenced by PGE2; both P4 and PGE2 hormones appeared to act independently. P4 did not affect *IGF1* expression, which was upregulated by PGE2, however, it suppressed expression of *IGF2*, also in the presence of PGE2. Similarly, P4 did not affect PGE2 synthase (*PTGES*), but in the presence of PGE2 it increased *PTGER2* levels and, regardless of the presence of PGE2, suppressed expression of *PTGER4*. Our results indicate a reciprocal regulatory loop between PGE2 and P4 during canine *in vitro* decidualization: whereas P4 may be involved in regulating PGE2-mediated decidualization by regulating the availability of its receptors, PGE2 regulates PGR levels in a manner dependent on PTGER2 and -4.

Key words: Canine (*Canis lupus familiaris*) decidualization, Dog uterine stromal (DUS) cells, Extracellular matrix (ECM), Pregnancy

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The establishment and maintenance of pregnancy requires a precise and orchestrated interplay between fetal trophoblast and maternal tissues, involving dynamic changes of the hormonal axis and species-specific morpho-functional changes in the uterus. In this context, during early pregnancy uterine immune, epithelial and stromal cells interact with each other, facilitating the successful onset of pregnancy (reviewed in [1, 2]). Consequently, in species exhibiting invasive types of placentation, maternal stroma-derived decidual cells develop. This species-specific tissue remodeling is referred to as decidualization, and is one of the most essential events at the beginning of pregnancy in rodents, carnivores and humans [3–6]. In rodents [6] and dogs [7, 8] maternal decidual cells of mesenchymal origin are formed in response to the presence of implanting blastocysts, whereas in humans decidua is spontaneously formed during every reproductive cycle in a progesterone (P4)-dependent manner (reviewed in [9]). Contrasting with the haemochorial type of placentation, and peculiar to the endotheliochorial type of placentation observed in carnivores including the dog, decidual cells together with maternal vascular endothelium can escape the proteolytic activity of trophoblast. Considering the importance of P4 in the maintenance of pregnancy, in the dog decidual cells are the only cells expressing the nuclear progesterone receptor (PGR) [10, 11], which is a species-specific feature. Despite being devoid of its own steroidogenic activity, due to the presence of PGR in its maternal component the canine placenta can respond to circulating luteal P4. Interfering with its function alters feto-maternal communication, leads to prepartum PGF2α synthesis, and terminates pregnancy in dogs (reviewed in [5, 12]). The central role of decidual cells in canine pregnancy becomes apparent when PGR-antagonists are applied, unequivocally leading to termination of pregnancy [10]. Clearly, thus, in agreement with the main goal of our studies, detailed investigations are needed into the decidualization process in the dog to improve its understanding, to increase clinical opportunities for more targeted management of pregnancies, and to control reproductive processes in this species.

At present, in the dog the development of decidual cells is still not well understood. Therefore, recently, we established a cellular *in vitro* model of canine decidualization using uterine stromal cells isolated from naturally estrogenized early diestric bitches [13]. Taking it further, a DUS (dog uterine stromal) cell line was generated [7]. DUS cells were morphologically and biochemically characterized, becoming a reliable model for investigating *in vitro* decidualization

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in this species [7]. We also established that prostaglandin (PG) E2 is capable of inducing the expression of canine decidualization markers in DUS cells, such as prolactin receptor (PRLR), PGR, PGE2-synthase (PTGES) and PGE2 receptor 4 (PTGER4/EP4) [7]. In this, similarities were found between the early decidualization processes in dogs and those in rodents and humans [14–16].

In fact, *in vivo* as well the PGE2-system appears to be involved in the decidualization process in the dog as it was found in epithelial and stromal compartments of the early pregnant uterus [8], and PGE2-synthesizing PTGES is abundantly expressed in free-floating blastocysts [8]. The potential involvement of particular PGE2 receptors in the underlying processes remains, however, to be shown. Due to dependence of the decidualization process on cAMP and/or its inducing stimuli [7, 13, 17], the present study was based on the hypothesis that PTGER2/EP2 and PTGER4/EP4 are involved in the underlying processes. Both PTGER2 and -4 act via the cAMP/PKA pathway [18]. Canine *in vivo* early decidualization is accompanied by extracellular matrix (ECM) remodeling [19]. This involves, e.g., the expression of fibronectin (FN) 1, extracellular matrix protein 1 (ECM1) and tissue inhibitor of metalloproteinases (TIMP) 2 and -4 [19, 20]. Furthermore, implantation and trophoblast invasion are also associated with strong structural changes in the uterus, characterized by modulation of collagen expression levels [19]. The contribution of the decidual cell compartment to matrix remodeling in the dog during both *in vitro* and *in vivo* decidualization remains, however, to be determined. This applies also to the PGE2-dependent mechanisms. So far, we have shown that primary DUS cells secrete increased amounts of proteins during cAMP-mediated decidualization [13]. Similarly, in mice, stromal cell lines secrete basement membrane components [21]. Interestingly, in both pregnant and non-pregnant animals the canine uterus is physiologically exposed to high P4 concentrations (see reviews in [5, 12, 22, 23]). P4 profiles and concentrations are similar in both situations [24], however, they do not lead to spontaneous decidualization as observed in humans. Possible interactions between PGE2 and P4-dependent mechanisms are indicated due to the PGE2-dependent induction of PGR expression in DUS cells *in vitro* [7]. These synergistic effects and the involvement of the respective PGE2-receptors in the underlying mechanisms need to be investigated.

Here, we utilized our proven *in vitro* decidualization model with immortalized DUS cells to fill existing knowledge gaps. The main goals of the project were: i) to investigate the potential involvement of PTGER2/4 in PGE2-dependent decidualization, ii) to assess the effects of cAMP and PGE2 on matrix remodeling, and iii) to demonstrate the potential synergistic effects of PGE2 and P4 during canine decidual cell formation.

Material and Methods

Cell culture and in vitro experiments with DUS cells

The DUS cell line was utilized for the present study [7]. The handling and stimulation of the cell culture followed the basic procedure as previously published [7, 13]. Briefly, after trypsinization and harvesting, cells were transferred into 6-well plates (TPP Techno Plastic Products AG, Trasandingen, Switzerland) at a concentration of 1×10^5 cells per well. For immunofluorescence (IF) staining, sterile glass cover slips were placed into the well, then cells were seeded onto them to allow adhesion to take place. After transferring the cells into the plates, prior to treatments, they were kept for 24 h under standard culture conditions (i.e., 37°C, 5% CO_2 in air, in a humidified incubator) in fresh cell culture medium consisting of DMEM-High Glucose (Bio Concept, Allschwil, Switzerland), pH 7.2–7.4, with 10% heat-inactivated FBS (Fetal Bovine Serum; Thermo Scientific AG, Reinach, Switzerland), 100 U/ml penicillin and 100 µg/ml streptomycin (PAN Biotech, Aidenbach, Germany) and 1% ITS (Insulin-Transferrin-Selenium; Corning from Thermo Scientific AG). The standard decidualization protocol with N6,2′-O-dibutyryladenosine-3′,5′-cyclicmonophosphate (dbcAMP, referred further to as cAMP) was applied as previously described [7, 13]. In short, after washing with PBS, cells were incubated with stimulation medium (cell culture medium with 0.1% bovine serum albumin (BSA) instead of FBS). Decidualization was induced with 0.5 mM cAMP (D0627, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) during 72 h [7, 25]. Moreover, DUS cells were incubated (i.e., decidualized) for 72 h with 10 µM PGE2 (P0409, Sigma-Aldrich) in stimulation medium, while stimulation medium without additives was used for a control. Both cAMP and PGE2 concentrations were derived from our previous studies [7, 13]. DUS cells were also incubated with stimulation medium that contained increasing dosages of P4 (P8783, Sigma-Aldrich) (0.01 µM = 10^{-8} M, 0.1 µM = 10^{-7} M, 1.0 µM = 10^{-6} M) alone or in combination with 10 µM PGE2. Selective blockers of PGE2 receptors (functional inhibition) from Cayman Chemical, Ann Arbor, MI, USA, were used targeted against PTGER2/EP2 (PF-04418948) and PTGER4/EP4 (GW-627368X). They were applied at increasing dosages (0.1 µM, 1.0 µM, 10 µM) in stimulation medium.

At least three consecutive experiments were performed for all cell culture experiments. The DM IL LED Fluo (Leica Microsystems CMS GmbH, Wetzlar, Germany) was used as an inverted bright field microscope and a LeicaDMI 6000B device served as the fluorescence microscope.

RNA isolation, reverse transcription (RT) and semi-quantitative (TaqMan) PCR

After the standard cell culture incubation period of 72 h, DUS cells were washed with cold PBS and harvested with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Detailed information about the following steps has been published before [13, 19, 26]. In short, from each sample 10 ng total RNA were used for RQ1 RNase-free DNase treatment (Promega, Dübendorf, Switzerland). This was followed by the use of the High Capacity cDNA Reverse Transcription Kit including RNase Inhibitor (Applied Biosystems from Thermo Fisher Scientific). Amplification of cDNA was performed according to the supplier's protocol with the TaqMan PreAmp Master Mix Kit (Applied Biosystems). TaqMan PCR was run in duplicates with the Fast Start Universal Probe Master (ROX) (Roche Diagnostics AG, Rotkreutz, Switzerland) in an automated fluorometer (ABI PRISM 7500 Sequence Detection System, Applied Biosystems). Controls consisted of running experiments with autoclaved water instead of cDNA and genomic DNA contamination was checked by the so-called RT-minus control [26, 27]. Table 1 presents a list of all TaqMan systems used for the present study. In-house designed

Table 1. List of all TaqMan systems used for semi-quantitative RT-PCR

Primer	Accession number		Primer Sequence	Product length (bp)
Extracellular Matrix Protein 1 *(ECM1)*	XM_845921.4	Forward	5′-CAG TCT GGC TTC TCC CAC CTT A-3′	99
		Reverse	5′-GCG GTT TGT GTG GCT GTG A-3′	
		TaqMan Probe	5′-AGA CTA GAT ATT CCC GCT GCT GCC GCT-3′	
Connexin 26 *(CX26/GJB2)*	AJ439693.1	Forward	5′-CCA CTA CTT CCC CAT CTC TCA CA-3′	98
		Reverse	5′-TCC GGT AGG CGA CAT GCA T-3′	
		TaqMan Probe	5′-CCG ACT CTG GGC TCT GCA GCT GAT C-3′	
Connexin 43 *(CX43/GJA1)*	AY462223	Forward	5′-AAA AGA GAA CCC TGC CCT CAT C-3′	91
		Reverse	5′-AGG ACA CGA CCA GCA TGA AGA-3′	
		TaqMan Probe	5′-ACT GCT TCC TCT CTC GCC CCA CG-3′	
Fibronectin 1 *(FN1)*	XM_014110981	Forward	5′-CAC GCC GAA CTA CGA TGC-3′	95
		Reverse	5′-TGC GAT ACA TGA CCC CTT-3′	
		TaqMan Probe	5′-AAG TTT GGA TTT TGC CCC ATG GCC-3′	
Laminin alpha 2 *(LAMA2)*	XM_014113700.1	Forward	5′-AAA CCG GCT CAC GAT TGA G-3′	99
		Reverse	5′-AGT TGA ACG GTG GCG AAG T-3′	
		TaqMan Probe	5′-CCT GCT CTT CTA CAT GGC TCG GAT CAA-3′	
Glyceraldehyd 3-phosphate dehydrogenase (*GAPDH*)	AB028142	Forward:	5′-GCT GCC AAA TAT GAC GAC ATC A-3′	75
		Reverse:	5′-GTA GCC CAG GAT GCC TTT GAG-3′	
		TaqMan Probe	5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	
Progesterone receptor *(PGR)*	NM_001003074	Forward:	5′-CGA GTC ATT ACC TCA GAA GAT TTG 1 1 1-3′	113
		Reverse:	5′-CTT CCA TTG CCC TTTTAA AGA AG A-3′	
		TaqMan Probe	5′-AAG CAT CAG GCT GTC ATT ATG GTG TCC TAA CTT-3′	
Prostaglandin E2 synthase *(PTGES)*	NM_001122854	Forward:	5′-GTC CTG GCG CTG GTG AGT-3′	89
		Reverse:	5′-ATG ACA GCC ACC ACG TAC ATC-3′	
		TaqMan Probe	5′-TCC CAG CCTTCC TGC TCT GCA GC-3′	
Prostaglandin E2 receptor 2 *(PTGER2/EP2)*	AF075602	Forward:	5′-CAC CCT GCT GCT GCT TCT C-3′	78
		Reverse:	5′-CGG TGC ATG CGG ATG AG-3′	
		TaqMan Probe	5′-TGC TCG CCT GCA ACTTTC AGC GTC-3′	
Prostaglandin E2 receptor 4 *(PTGER4/EP4)*	NM_001003054	Forward:	5′-AAA TCA GCA AAA ACC CAG ACT TG-3′	96
		Reverse:	5′-GCA CGG TCT TCC GCA GAA-3′	
		TaqMan Probe	5′-ATC CGA ATT GCT GCT GTG AAC CCT ATC C-3′	
Prolactin receptor *(PRLR)*	HQ267784	Forward:	5′-GGA TCT TTG TGG CCG TTC TTT-3′	92
		Reverse:	5′-AAG GAT GCA GGT CAC CAT GCT AT-3′	
		TaqMan Probe	5′-ATT ATG GTC GTA GCA GTG GCT TTG AAA GGC-3′	
Tissue inhibitor of matrix metalloproteinase-2 *(TIMP2)*	AF188489	Forward	5′-CAT AGG TAC CAG ATG GGC TGT GA-3′	95
		Reverse	5′-CAG TCC ATC CAG AGG CAC TCA-3′	
		TaqMan Probe	5′-TGA TCC CGT GCT ATA TCT CGT CTC CGG-3′	
Tissue inhibitor of matrix metalloproteinase-2 *(TIMP4)*	NM_001314106	Forward	5′-CTG TGG CTG CCA AAT TAC CA-3′	103
		Reverse	5′-CCC ATA GAG CTT CCG TTC CA-3′	
		TaqMan Probe	5′-ACC ATC TCA GCC CCT AAC GAG TGC CTC-3′	
Cyclophilin *(PPIA)*	XM_843327.1	Applied Biosystems, prod nr. Cf03986523_gH		92
Insulin-like growth factor 1 *(IGF1)*	NM_001313855.1	Applied Biosystems, prod nr. Cf02627846_m1		104
Insulin-like growth factor 2 *(IGF2)*	NM_001195403	Applied Biosystems, prod nr. Cf02647136_m1		126
ActinB	NM_001003349.1	Applied Biosystems, prod nr. Cf03023880_g1		121
Collagen, type 1, alpha 1 *(COL1A1)*	NM_001003090	Applied Biosystems, prod nr. Cf02741575_mH		97
Collagen, type 3, alpha 1 (*COL3A1)*	XM_845916	Applied Biosystems, prod nr. Cf02631366_m1		98
Collagen, type 4, alpha 1 *(COL4A1)*	XM_014106444	Applied Biosystems, prod nr. Cf02696157_mH		82

systems were purchased from Microsynth (Balgach, Switzerland) and were validated by a probe efficiency test as previously described [26, 27]. FAM- and TAMRA-labeled, pre-designed, commercially available TaqMan systems were ordered from Applied Biosystems. Three reference genes were used for normalization: *glycerinaldehyd-3-phosphat-dehydrogenase* (*GAPDH*), *cyclophilin A* and *actinB*. The calculation of fold gene expression values was as previously reported [26]. Briefly, qPCR was performed in duplicates and the average Ct values were determined by calculating the arithmetic means. The average Ct values were used to calculate ΔCt. Next, the sample with the lowest expression was selected as the calibrator. This was followed by calculating ΔΔCt and the fold gene expression. The statistical analysis was performed with log transformed values.

IF staining, CellProfiler and evaluation of data

IF staining was performed as previously described [7, 28]. In short, following the standard cell culture incubation period of 72 h, the cells adhering to a sterile glass cover slip were fixed by adding formalin to the incubation medium to a final concentration of 2%, for 10 min at 37°C. Following glycine treatment (5 min) and blocking with goat serum (30 min), the primary antibody was incubated for 2 h at ambient temperature. The list of antibodies and their specific dilutions can be found in Table 2. Following incubation with the secondary antibody (equipped with either Alexa Fluor 594 or Alexa Fluor 466 dyes) in combination with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) used at a concentration of 1:1000, the glass cover slip was mounted using Glycergel® (Sigma-Aldrich) on a microscope slide and representative pictures were taken with the LeicaDMI 6000B fluorescence microscope equipped with a Leica DFC360FX camera. Controls were used as previously published [7, 10, 13], i.e., staining with only primary or secondary antibodies was performed as well as omitting any antibody to check for autofluorescence. Additional controls consisted of vimentin (mesenchymal cell marker), and SV40Tag (nuclear marker of immortalization) staining.

Next, the open-source software CellProfiler 3.0.0 [29] was used to determine the IF intensity of selected ECM proteins and to count the number of cells per field of view. Following IF co-staining [i.e., DNA staining with DAPI in addition to ECM-proteins staining against aminin alpha 2 (LAMA2), extracellular matrix protein 1 (ECM1), connexin 43 (CX43) or collagen type 4 (COL4)], the LeicaDMI 6000B fluorescence microscope equipped with a Leica DFC360FX camera was used to take 6 pictures of random view fields with a 63× dry magnification lens. At least 3 consecutive experiments were investigated independently. Software settings of CellProfiler 3.0.0 were adjusted according to either nuclei count or to ECM signal detection as described before [29]. DAPI staining was used to count cell nuclei per field of view. To ensure that the same number of cells was investigated in both conditions, nuclei counts were summed for each condition (i.e., the control well and the cAMP-stimulated well) and experiment individually and adjusted to each other by omitting single pictures for further analysis. A count of at least 200 cells and a difference between control and cAMP condition of +/– 10 cells were the minimum requirements for the adjustment. Following this, the remaining pictures were analyzed for the mean fluorescence intensity of ECM target protein (i.e., LAMA2, ECM1, CX43 or COL4). At least three consecutive experiments were independently evaluated from each other.

Statistics

Statistics were performed with GraphPad 3.06 Software (GraphPad Software, San Diego, CA, USA) for the normalized TaqMan ΔΔCt values and the mean IF intensity. For all performed statistics the data had to pass the statistical assumptions for normality and equality of variances. TaqMan experiments were evaluated with a parametric one-way ANOVA. In the case of $P < 0.05$ (i.e., difference among values was considered significant), the Tukey-Kramer multiple comparisons post-test was performed. The mean IF intensity was evaluated through an unpaired two-tailed Student's *t*-test. Numerical data are presented as geometric means Xg +/– geometric standard deviation (SD).

Results

cAMP, but not PGE2, modulates the expression of ECM-related factors during in vitro decidualization of DUS cells

The mesenchymal character of DUS cells presented previously [7] was confirmed herein (Fig. 1A, B). They exhibit vimentin staining and show stable genomic incorporation of the pSV40Tag (marker of immortalization) in their genome. Next, DUS cells were decidualized *in vitro* using 0.5 mM cAMP or 10 μM PGE2 as indicated in Material and Methods, and the expression of selected ECM-related factors was evaluated on the mRNA level for the following genes: *COL1*, *COL3*, *COL4*, *ECM1*, *LAMA2*, *FN1*, *CX26*, *CX43*, *TIMP2* and *TIMP4*. Whereas *LAMA2* expression was significantly suppressed during cAMP-mediated decidualization ($P < 0.001$), the mRNA levels of *ECM1*, *CX43* and *COL4* were upregulated ($P < 0.001$) (Fig. 1C),

Table 2. List of primary and secondary antibodies used for immunofluorescence staining

Antibody	Company	Reference Number	Host	Dilution
Collagen IV (COL4)	Abcam	ab6586	rabbit polyclonal	1:300
Laminin 2 alpha (LAMA2)	Bioss Antibodies	bs-8561R	rabbit polyclonal	1:100
Connexin 43 (CX43/GJA1)	Abcam	ab11370	rabbit polyclonal	1:400
Extracellular Matrix Protein 1 (ECM1)	Proteintech	11521-1-AP	rabbit monoclonal	1:100
Vimentin	Abcam	ab92547	rabbit monoclonal	1:500
SV40T-antigen (SV40Tag)	Abcam	ab16879	mouse monoclonal	1:500
Alexa fluor 594 goat anti-rabbit IgG (H+L)	Invitrogen	A11037	goat	1:100
Alexa fluor 488 goat anti-mouse IgG (H+L)	Invitrogen	A11029	goat	1:100

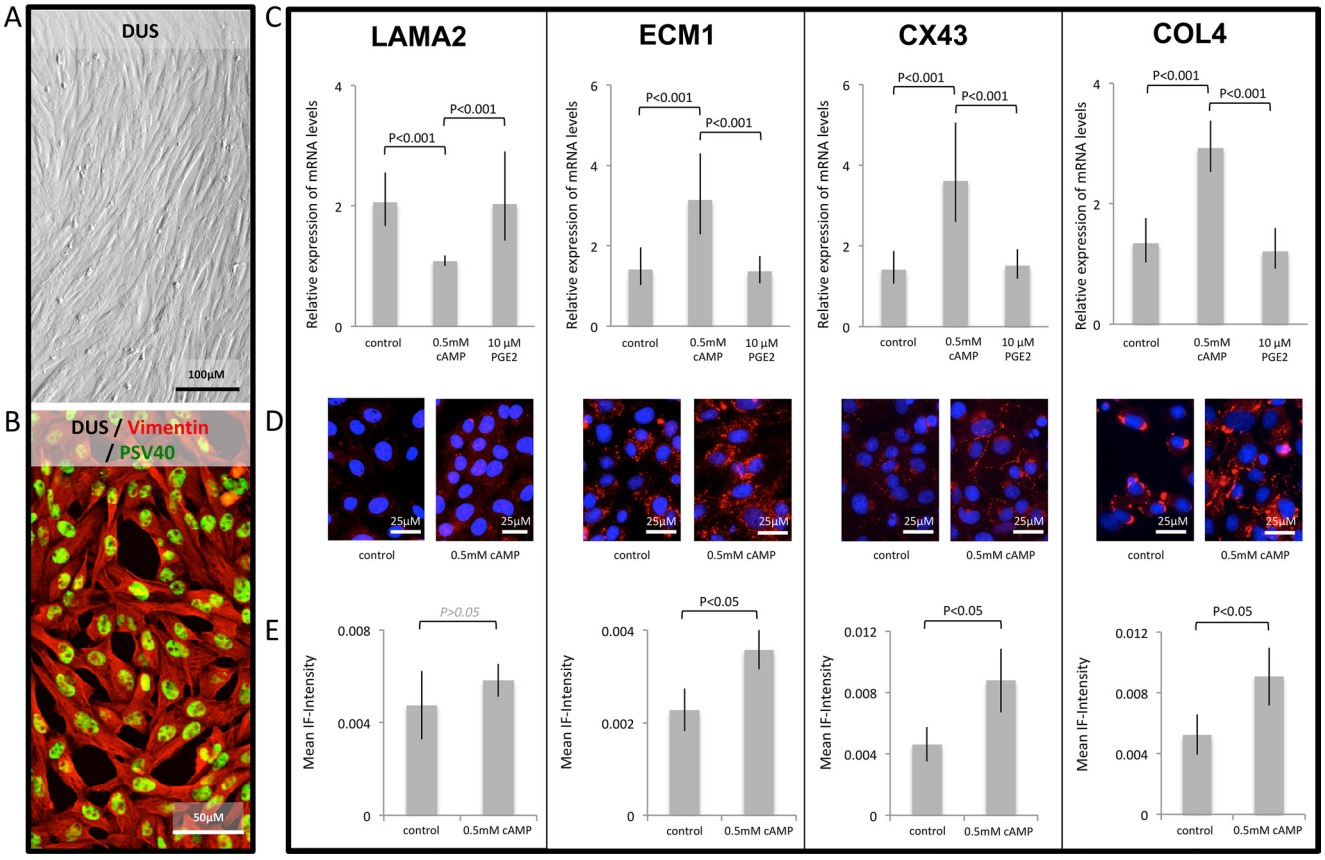

Fig. 1. Expression of selected extracellular matrix factors during cAMP- and prostaglandin (PG) E2-mediated *in vitro* decidualization of dog uterine stroma (DUS) cells. (A) Morphological appearance of DUS cells. (B) Expression of vimentin (mesenchymal cell marker; red color) and pSV40 Tag (marker of immortalization; green color) was verified by immunofluorescence (IF). (C–E) Cells were treated either with 10 µM PGE2 or with 0.5 mM cAMP over a time course of 72 h. (C) Relative mRNA expression of selected extracellular matrix (ECM) factors and of CX43. A parametric one-way ANOVA was applied followed by the Tukey-Kramer multiple comparisons post-test. Numerical data are presented as geometric means Xg ± geometric standard deviation (SD). P-values < 0.05 were considered significant and are indicated. (D) Representative IF staining of laminin alpha 2 (LAMA2), extracellular matrix protein 1 (ECM1), connexin 43 (CX43) and collagen type 4 (COL4) in control and cAMP-stimulated DUS cells (target protein = red color, DAPI = nuclear staining, blue color), and (E) corresponding quantification of selected protein expression as determined by mean IF intensity. Statistical differences were determined by an unpaired two-tailed Student's *t*-test. Numerical data are presented as geometric means Xg ± SD. P-values are indicated.

and were significantly more highly expressed ($P < 0.001$) than in PGE2-treated cells. PGE2 did not show any significant effects on the expression of ECM-related target genes ($P > 0.05$). Neither cAMP nor PGE2 had any effect on the expression of *COL1*, *COL3*, *FN1*, *CX26*, or *TIMP2* and *TIMP4* ($P > 0.05$, data not shown).

In the next step, the ECM-associated genes that were significantly altered at the mRNA level during cAMP-mediated decidualization were further analyzed at the protein level by measuring the mean IF intensity. In Fig. 1D, representative IF pictures are shown of DUS cells treated with or without cAMP and stained against LAMA2, ECM1, CX43 or COL4. For every experiment, 6 pictures were taken from randomly chosen areas and submitted to CellProfiler and the mean IF intensity was determined. Whereas the mean IF intensity of LAMA2 did not differ significantly ($P > 0.05$) between control and cAMP conditions, the intensity of ECM1, CX43 and COL4 increased following the treatment ($P < 0.05$) (Fig. 1E).

PGE2-mediated expression of PRLR and PGR in DUS cells is PTGER2/EP2- and PTGER4/EP4-dependent

DUS cells were decidualized *in vitro* with PGE2 following the application of increasing dosages of specific functional blockers of PTGER2 or PTGER4. The gene expression level of *PRLR*, *PGR* as well as of *PTGES* and *PTGER2* and *-4* was assessed (Fig. 2). Both blockers significantly abolished the stimulatory effects of PGE2 on *PRLR* and *PGR* expression during the decidualization process. The *PRLR* expression was significantly suppressed ($P < 0.05$) with 1.0 and 10 µM of PTGER2 blocker, and decreased gradually, reaching the lowest expression in response to 10 µM of the PTGER4 blocker ($P < 0.001$) (Fig. 2A, B). Similarly, *PGR* was significantly suppressed in response to 1.0 and 10 µM of PTGER2-blocker ($P < 0.01$ and $P < 0.001$, respectively). When PTGER4 blocker was applied, the *PGR* mRNA levels decreased gradually by $P < 0.05$, $P < 0.01$ and $P < 0.001$, in response to 0.1, 1.0 and 10.0 µM PTGER4 blocker, respectively (Fig. 2A, B). Neither of the blockers had a significant

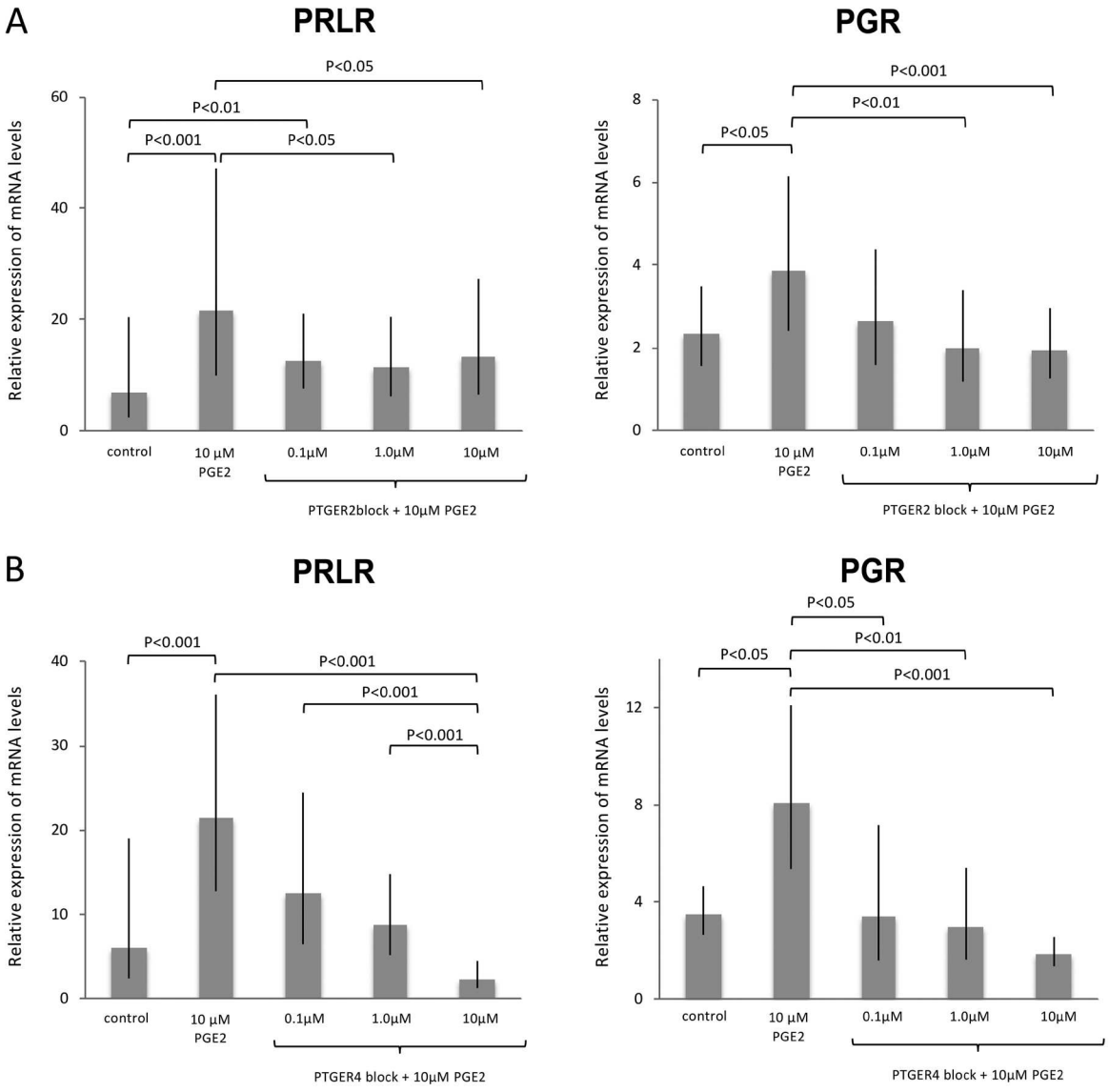

Fig. 2. Functional blocking of PGE2 receptor 2 (PTGER2/EP2) and PGE2 receptor 4 (PTGER4/EP4) in decidualizing dog uterine stroma (DUS) cells. Expression of prolactin receptor (PRLR) and progesterone receptor (PGR) as determined by real time (TaqMan) PCR. Selective PTGER2 (A) or PTGER4 (B) blockers were used as described in Material and Methods. A parametric one-way ANOVA was applied followed by the Tukey-Kramer multiple comparisons post-test. Numerical data are presented as geometric means Xg ± geometric standard deviation (SD). P-values < 0.05 were considered significant and are indicated.

effect on expression of the PGE2 system, i.e., *PTGES* and the two receptors (*PTGER2/4*) ($P > 0.05$, data not shown).

The effects of progesterone (P4) on expression of decidualization markers

DUS cells were treated for 72 h with cAMP or PGE2 (positive controls) or with increasing P4 concentrations in the absence (left-hand side) or presence (right-hand side) of PGE2 (Figs. 3, 4). The expression of *PRLR*, *PGR*, *IGF1* and *IGF2* was investigated (Fig. 3), as well as of the PGE2-system, i.e., *PTGES*, *PTGER2* and *PTGER4* (Fig. 4). Whereas cAMP significantly increased the mRNA levels of *PRLR*, *PGR* and *IGF1* ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively), the expression of *IGF2* was suppressed by cAMP ($P < 0.001$) (Fig. 3).

PRLR and *PGR* gradually increased significantly in response to increasing P4 concentrations, reaching the highest concentrations in response to 10^{-6} mM P4. The stimulatory effects of P4 on its own receptor (*PGR*) were stronger than that of cAMP ($P < 0.05$ at 10^{-6} mM P4). Interestingly, while *IGF1* was not affected by treatment with P4, *IGF2* was significantly suppressed in response to increased concentration of P4 ($P < 0.001$).

When treated with PGE2, *PRLR* ($P < 0.05$), *PGR* ($P < 0.05$) and *IGF1* ($P < 0.001$) were significantly elevated compared with

non-treated controls. These effects were not altered for either of these genes in the presence of P4 in the stimulation medium ($P > 0.05$). This was, however, not the case for *IGF2*. Thus, whereas it was not affected by PGE2 ($P > 0.05$) when applied at highest concentration (10^{-6} mM), P4 significantly suppressed *IGF2* expression as well in the presence of PGE2 ($P < 0.001$) (Fig. 3).

As for the PGE2-system, the expression levels of *PTGES*, *PTGER2* and *PTGER4* were upregulated in response to cAMP ($P < 0.05$ for *PTGES* and *PTGER2*, and $P < 0.001$ for *PTGER4*), whereas PGE2 increased the mRNA levels of its own synthase *PTGES* ($P < 0.05$) and of *PTGER4* ($P < 0.05$). With regard to the effects of P4, it significantly suppressed the expression of *PTGER4* even when used at the lowest concentrations ($P < 0.05$ and $P < 0.001$, for 10^{-7} mM and 10^{-8} mM, respectively) (Fig. 4), but no P4 effects were noted for *PTGES* and *PTGER2* mRNA levels ($P > 0.05$). However, when used in co-treatment, PGE2 together with P4 applied at the highest concentration (10^{-6} mM) had a positive effect on *PTGER2* ($P < 0.001$). As also revealed in co-treatment experiments, and already shown in single treatments, P4 suppressed the expression of *PTGER4* regardless of the presence of PGE2 in the stimulation medium ($P < 0.01$ and $P < 0.001$ for 10^{-7} and 10^{-6} mM P4).

Discussion

Investigating the development and function of canine decidual cells appears to be one of the most important tasks required for better understanding of the physiology of canine pregnancy. Morphologically, in the dog, decidualization starts at the time of embryo implantation (around day 17 of embryonal life) with strong endometrial proliferation being associated with histological changes in the sub-epithelial stromal compartments [7]. The vimentin-positive, interstitial stromal cells become larger and rounded, undergoing morpho-functional decidualization [7]. These round cells with ovoid nuclei and dense chromatin were also previously found in the early pregnant canine uterus as NEP/CD10-negative cells sharing morphological resemblance with predecidual cells of women [30]. NEP/CD10 (neutral endopeptidase, neprylisin, enkephalinase) is a multifunctional protein and metalloprotease normally characteristic of uterine stromal cells [30]. Similar to humans, in the dog also stromal decidualization can be recapitulated *in vitro* and is associated with increased secretory activity of decidualizing cells [7, 13]. Here, making use of the recently generated DUS cell line [7], and using our previously established cAMP- and PGE2-mediated approaches [7, 13], *in vitro* decidualization-associated expression of selected ECM-modulating compounds and of CX43 was investigated. All factors were clearly detectable in DUS cells during decidualization protocols. ECM1 and COL4 proteins were significantly increased in cAMP-stimulated cells. In addition to its function in extracellular matrix formation, ECM1 has been shown to be involved in cellular differentiation, maturation and proliferation [31]. Additionally, it exhibits the potential to reduce proteolytic activities of some metalloproteinases, e.g., MMP9 [32]. Its involvement in regulating uterine and placental functions in the dog was suggested recently [19]. COL4 is a component of the basal lamina. Its increased expression in decidualized DUS cells implies the mesenchymal-epithelial decidual transition of uterine stromal compartments described previously for humans [33]. Thus, while continuously expressing vimentin and characterized by high levels of alpha-smooth muscles actin (αSMA) [7, 13], canine decidual cells appear to exhibit some epithelioid characteristics previously known from other cellular models of decidualization. Interestingly, in human decidualized stromal cells, elevated levels of COL4 were associated with decreased expression of NEP/CD10 [33], a further sign of epithelioid decidual transition. Also, similar to the situation observed in humans, we found an increase in connexin 43 (CX43) levels in decidualized cells, both at the mRNA and protein levels. This seems to be an important finding. As an adhesion and gap junction molecule, CX43 is also critical for differentiation of endometrial stromal cells in other species, e.g., in humans [34, 35]. Disruption of its function induces inflammatory mediators and leads to apoptosis of stromal cells [34, 35]. Cumulatively, our findings regarding ECM-related factors and CX43 indicate similarities between canine decidualization and the decidual mesenchymal-epithelial transition observed in humans. Corroborating these findings, recently, as revealed by a transcriptomic approach, we found a positive correlation between sets of genes expressed in the preimplantation canine uterus and genes expressed in the human uterus during the window of implantation [20]. The number of matching genes was higher than that detected in comparison with other domestic animal species (horse, pig and cattle), displaying a non-invasive placentation type. The close match between canine and human preimplantation endometrium was interpreted as a result of the ongoing decidualization process. Nevertheless, it needs to be re-emphasized that, in contrast with the situation observed in humans where decidualization is spontaneously cyclically induced by P4, in dogs as in rodents, decidualization is induced by uterine exposure to embryos and embryo-maternal contact [7, 20].

Contrasting with the cAMP-mediated approach, as presented herein, PGE2-driven decidualization did not affect the expression of investigated ECM components. This is an interesting observation. Thus, although revealing decidualization capacities, PGE2 does not seem to be strongly involved in regulating matrix assembly nor the expression of CX43 in canine decidual cells.

Adding to the previous facts regarding the role of PGE2 during canine decidualization, in the present study, we were able to demonstrate that the PGE2-driven activation of *PGR* and *PRLR* expression is modulated by both cAMP/PKA-dependent PGE2 receptors, i.e., PTGER2 and PTGER4. The functional suppression of their activity diminished the PGE2-driven expression of both *PRLR* and *PGR*. By including a mechanistic insight related to the PTGER2 and -4 involvement in regulating *PGR* expression, the present study further emphasizes the previously postulated [7] interaction between PGE2 and P4-dependent mechanisms regulating canine uterine function during pregnancy. These species-specific regulatory features appear to be of translational importance for comparative research in other mammals. The mechanism of the possible amplification loop within the PGE2 system appears interesting and requires further investigations. Accordingly, PGE2 upregulates the expression of its own synthase (PTGES) and of the PTGER4 receptor, however, expression of the PGE2 system in decidualizing DUS cells treated with PTGER2/-4 blockers did not change significantly. This could be due to compensatory mechanisms existing between the two PGE2 receptors.

Although still not fully understood, the role of decidual cells in

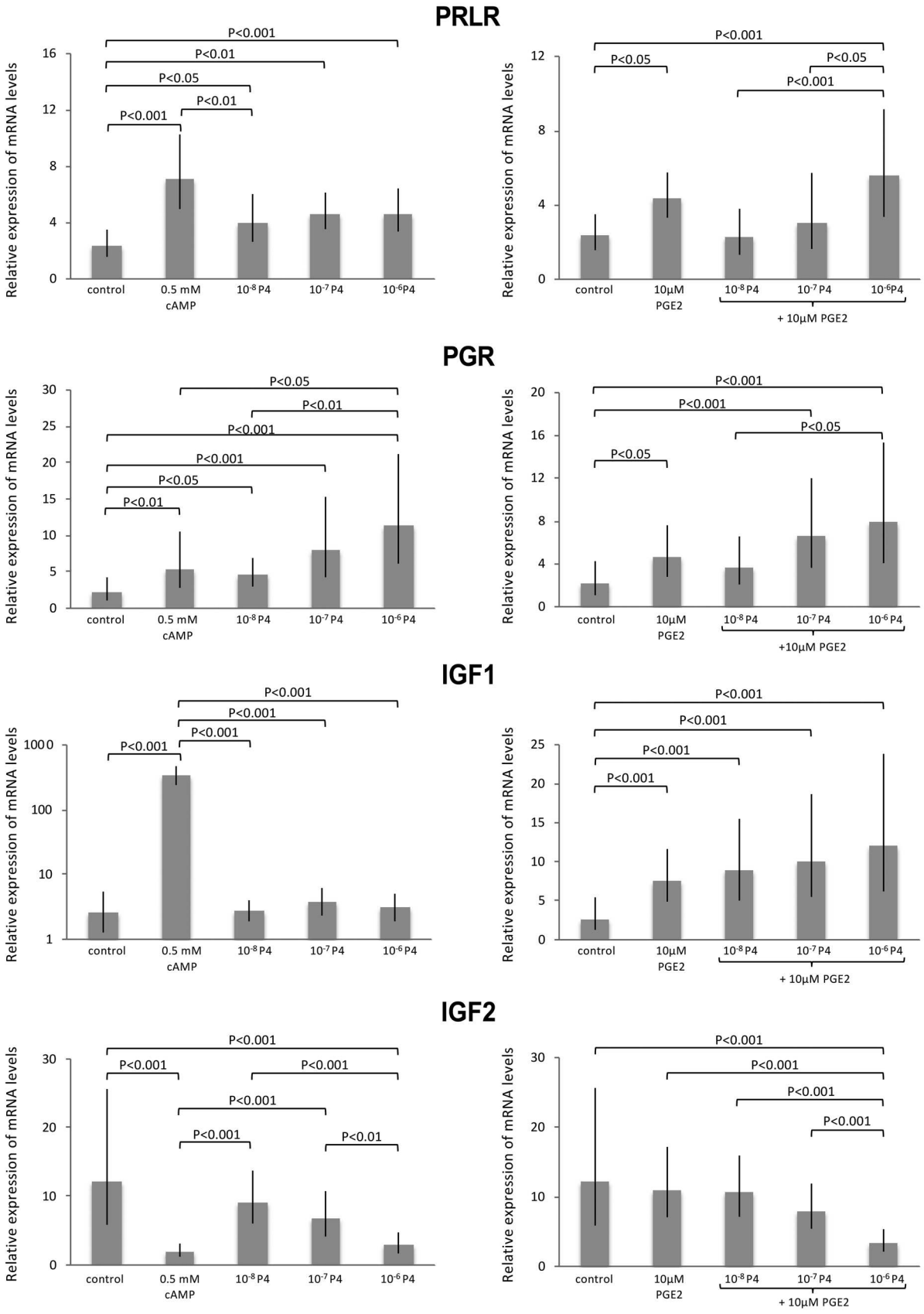
PRLR
Relative expression of mRNA levels
control
0.5 mM cAMP
10-8 P4
10-7 P4
10-6P4
10μM PGE2
+ 10μM PGE2
PGR
IGF1
IGF2
P<0.001
P<0.01
P<0.05

Fig. 3.

Fig. 3. Gene expression of prolactin receptor (PRLR), progesterone receptor (PGR), insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) as determined by real time (TaqMan PCR). Dog uterine stroma (DUS) cells were stimulated with different dosages (10^{-6}, 10^{-7},10^{-8} mM) of progesterone (P4), alone or in the presence of 10 μM PGE2; 0.5 mM cAMP was used as a positive control. The treatment was applied for 72 h. A parametric one-way ANOVA was applied followed by the Tukey-Kramer multiple comparisons post-test. Numerical data are presented as geometric means Xg ± geometric standard deviation (SD). P-values < 0.05 were considered significant and are indicated. A logarithmic scale was used to present results for IGF1.

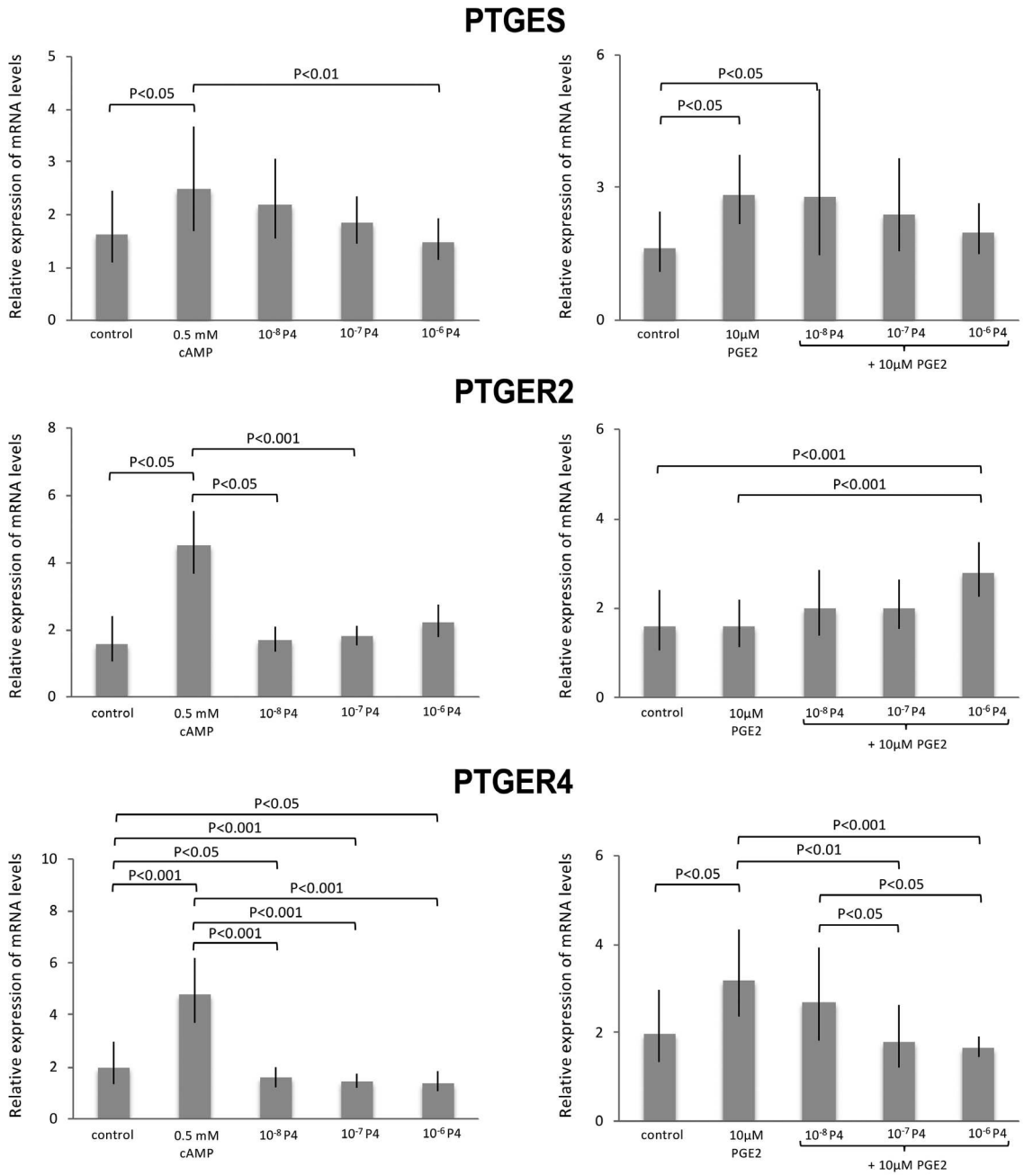

Fig. 4. Gene expression of PGE2-synthase (PTGES), PGE2 receptor 2 (PTGER2/EP2) and PGE2 receptor 4 (PTGER4/EP4) as determined by real time (TaqMan PCR). Dog uterine stroma (DUS) cells were stimulated with different dosages (10^{-6}, 10^{-7}, 10^{-8} mM) progesterone (P4), alone or in the presence of 10 μM PGE2; 0.5 mM cAMP was used as a positive control. The treatment was applied for 72 h. A parametric one-way ANOVA was applied followed by the Tukey-Kramer multiple comparisons post-test. Numerical data are presented as geometric means Xg ± geometric standard deviation (SD). P-values < 0.05 were considered significant and are indicated.

the dog is largely determined by their expression of the nuclear PGR, which plays an important role during maintenance of pregnancy [10, 36]. Thus, in the next step, by making use of DUS cells we were also able to investigate possible synergistic effects between P4 and PGE2 during *in vitro* decidualization. Such mechanisms exist for human stromal cells, in which PGE2 accelerates the P4-mediated decidualization [37]. Consequently, in our next experiments cells were treated with P4 alone or in co-treatment with PGE2. Both cAMP and PGE2 were used as positive controls, revealing increased expression of *PRLR*, *IGF1*, *PGR* and the PGE2-system following cAMP treatment. Whereas in our previous experiments *IGF2* expression, while displaying high variation, remained statistically unaffected, in the present experiments, it was clearly suppressed in cAMP-decidualized DUS cells. This was associated with very high IGF1 levels and could relate to yet undefined conditions, e.g., the culture medium or other experimentally-dependent variations of gene expression. However, it needs to be emphasized that, due to the restricted availability of canine-specific or cross-reacting antibodies, the present analysis was largely conducted at the messenger level. Until the expression of the respective proteins can be investigated, any final conclusion would be premature. As for PGE2, besides the above-mentioned positive effects on *PRLR* and *PGR*, PGE2 confirmed its capability to increase the expression of its own synthase (*PTGES*) and *PTGER4*. Additionally, *IGF1* expression was significantly upregulated. Interestingly, when used alone, P4 upregulated the gene expression of *PGR* and *PRLR*, thereby revealing a basic capability to modulate the expression of both decidualization markers. Notably, the effects of P4 on the expression of its own receptor were stronger than those exerted by cAMP, implying a functional enhancement loop between P4 and *PGR*. These effects were not affected by PGE2, and both hormones, i.e., P4 and PGE2, appeared to act independently. Similarly, P4 did not change *IGF1* expression, and neither did it influence the stimulatory effects of PGE2 on IGF1. In contrast, regardless of the presence of PGE2 in the stimulation medium, P4 diminished *IGF2* mRNA levels in a dose-dependent manner. Nevertheless, P4 appears to possess a regulatory capability on the PGE2-system, by modulating *PTGER2* and *-4* expression. Accordingly, when used in cotreatment with PGE2, at higher concentrations P4 upregulated *PTGER2* expression, and regardless of the presence of PGE2, diminished *PTGER4* mRNA levels. P4 thus appears to modulate the availability of *PTGER2* and *-4* in opposite directions.

Needless to say, taking into account the complexity of the decidualization process and the lack of the natural cell-to-cell interaction in an *in vitro* system lacking different cellular compartments, as in any *in vitro* study, the findings obtained from cell culture studies need to be verified *in vivo*.

Undoubtedly, being responsible for the maintenance of canine pregnancy, and initiation of the luteolytic cascade by targeting placental decidual cells, the P4/PGR system is an important player regulating canine uterine and placental functions. During decidualization, canine stromal cells appear to undergo mesenchymal-epithelial differentiation that is also typical of other decidualization models, as reflected in increased expression of COL4, ECM1 and strongly enhanced presence of CX43. Although P4 is considered to be a weaker spontaneous inducer of decidualization in the dog, our *in vitro* study suggests a direct involvement of P4-mediated pathways in canine decidualization by modulating the expression of several decidualization markers. Investigating them is certainly worth further attention as it could reveal important mechanisms underlying the regulation of canine embryo-maternal communication, determining the maintenance or termination of canine gestation and thereby bearing great clinical relevance.

Conflict of Interests: The authors declare that they have no cinflict of interests.

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